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Three of the Four Nucleocapsid Proteins of Marburg Virus, NP, VP35, and L, Are Sufficient To Mediate Replication and Transcription of Marburg Virus-Specific Monocistronic Minigenomes

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This paper describes the first reconstituted replication system established for a member of the *Filoviridae*, Marburg virus (MBGV). MBGV minigenomes containing the leader and trailer regions of the MBGV genome and the chloramphenicol acetyltransferase (CAT) gene were constructed. In MBGV-infected cells, these minigenomes were replicated and encapsidated and could be passaged. Unlike most other members of the order *Mononegavirales*, filoviruses possess four proteins presumed to be components of the nucleocapsid (NP, VP35, VP30, and L). To determine the protein requirements for replication and transcription, a reverse genetic system was established for MBGV based on the vaccinia virus T7 expression system. Northern blot analysis of viral RNA revealed that three nucleocapsid proteins (NP, VP35, and L) were essential and sufficient for transcription as well as replication and encapsidation. These data indicate that VP35, rather than VP30, is the functional homologue of rhabdo- and paramyxovirus P proteins. The reconstituted replication system was profoundly affected by the NP-to-VP35 expression ratio. To investigate whether CAT gene expression was achieved entirely by mRNA or in part by full-length plus-strand minigenomes, a copy-back minireplicon containing the CAT gene but lacking MBGV-specific transcriptional start sites was employed in the artificial replication system. This construct was replicated without accompanying CAT activity. It was concluded that the CAT activity reflected MBGV-specific transcription and not replication.

Marburg virus (MBGV) is the prototype member of the family *Filoviridae*, which belongs to the order *Mononegavirales*. MBGV causes a severe hemorrhagic disease in monkeys and humans that results in high fatality rates. The genomic RNA of MBGV is 19,108 nucleotides (nt) in length (EMBL nucleotide sequence database accession no. Z12132) (5) and is transcribed into monocistronic mRNA species encoding seven structural proteins (17, 27). These are a single surface protein (GP) inserted in the viral membrane (2, 37), two putative matrix proteins (VP40 and VP24), and the nucleocapsid proteins. In contrast to most rhabdo- and paramyxoviruses, which are known to possess three nucleocapsid proteins, filoviruses contain one additional protein that is associated with the core complex (1, 15). The four nucleocapsid proteins of MBGV are the nucleoprotein (NP) (3, 33), the L protein (28), and the viral proteins VP35 and VP30. NP and L are thought to be filovirus-specific homologues of the nucleoprotein and the polymerase subunit L of other nonsegmented negative-stranded (NNS) RNA viruses. As the second protein encoded in the genome, VP35 is presumed to be the P equivalent of filoviruses. However, VP35 is only weakly phosphorylated (unpublished data) and therefore differs from all P proteins of other NNS RNA viruses.

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In contrast to VP35, the fourth nucleocapsid protein of filoviruses (VP30), encoded by the fifth gene, is highly phosphorylated (reference 15 and unpublished data). The only NNS RNA viruses also known to possess an additional nucleocapsid protein (M2) are pneumoviruses (18), and the gene coding for M2 is located adjacent to the L gene. Recently, Collins and coworkers (8) determined that the M2 protein (also called the 22K protein) of respiratory syncytial virus (RSV) is essential for proper elongation of viral mRNAs in a reconstituted, cDNA-expressed RSV minigenome system. Furthermore, M2 was found to act as an antiterminator during viral transcription (22).

To date, little is known about the function of the different nucleocapsid proteins of MBGV. To obtain more information on the MBGV replicative cycle and the proteins which are involved in this process, an artificial replication system based on the vaccinia virus T7 expression system has been established. Such systems have been developed previously for various other NNS RNA viruses (9). Briefly, cDNAs of naturally occurring RNA minigenomes (6, 29, 30) or cDNAs of synthetic minigenomes containing leader and trailer regions of the respective viral genome and usually a reporter gene (chloramphenicol acetyltransferase [CAT], luciferase, or viral genes) are inserted in a transcription vector under the control of the T7 RNA polymerase promoter (10, 14, 21, 24, 32, 35, 38). Cells expressing the T7 RNA polymerase and the viral proteins essential for replication and transcription are transfected with the artificial minigenomic DNA which will be transcribed by the T7 RNA polymerase. If the minigenome is accepted as a template by the recombinant viral proteins, virus-specific transcription and replication will take place, thus mimicking the authentic viral replication complex. These artificial replication

systems are helpful tools for the analysis of *cis*- and *trans*-acting elements influencing RNA synthesis.

The present study describes the first reverse genetic system for filoviruses. The essential protein components of this system and the conditions under which transcription and replication take place have been determined.

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MATERIALS AND METHODS

Viruses and cell lines. The Musoke strain of MBGV, isolated in 1980 in Kenya (34), was grown in E6 cells, a Vero cell line clone (ATCC CRL 1586), as described by Mühlberger et al. (28). For T7 RNA polymerase expression, the recombinant vaccinia virus MVA-T7, which was grown in chicken embryo fibroblasts, was used (36).

Molecular cloning. (i) Cloning of nucleocapsid protein genes. cDNAs containing the open reading frame (ORF) of NP, VP35, VP30, and L were cloned into the T7 expression vector pTM1 (kindly provided by Bernhard Moss, National Institutes of Health). The ORF and parts of the nontranslated regions of all genes were amplified by reverse transcription-PCR (RT-PCR) with specific primers containing appropriate restriction sites. The amplified products were subsequently inserted into the *Eco*RI (for NP) or *Bam*HI (for VP35 and VP30) site of pTM1. The NP construct spans nt 98 to 2377 of the MBGV genome, VP35 spans nt 2939 to 4337, and VP30 spans nt 8863 to 9982; the resulting plasmids were designated pT/NP, pT/VP35, and pT/VP30, respectively. Cloning of the L gene involved synthesis of three PCR fragments. The first (nt 11473 to 13784) was flanked by a *Sac*I-*Not*I site at its 5′ end (mRNA sense) and a *Bam*HI site at its 3′ end and was cloned into the *Sac*I-*Bam*HI sites of the vector pGEM3Zf(+). This clone was designated pGEM/L1.1. The second fragment (nt 13334 to 15587) was inserted into pGEM/L1.1 by using the L-specific restriction sites *Cla*I and *Bam*HI. The resulting clone, spanning the first 4,408 nt of the L gene, was designated pGEM/L1. The third fragment (nt 15853 to 18898), amplified to contain an *Sph*I-*Not*I site, was inserted into the *Bam*HI and *Sph*I sites of clone pGEM/L1. The resulting clone, pGEM/L, contained the complete ORF of the L gene. For cloning of L into the vector pTM1, a *Not*I linker (Boehringer Mannheim) was ligated into the *Bam*HI site of pTM1 and the L ORF was inserted as a *Not*I fragment; the resulting clone was designated pT/L.

(ii) Construction of artificial MBGV minigenomes. The first 106 nt of the 3′ end of the MBGV genome (leader) were amplified by RT-PCR. For cDNA synthesis, 50 to 100 ng of viral RNA (vRNA) was incubated with a primer complementary to nt 2 to 32 of the vRNA (3′ end) and containing *Nco*I and *Sph*I restriction sites. RT was performed for 40 min at 42°C. The second primer for PCR was homologous to nt 78 to 106 of the vRNA and contained *Not*I and *Bam*HI restriction sites. The final concentration of both primers was 0.3 μM. PCR conditions were as follows: 35 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C. A 439-nt fragment of the 5′ end (trailer) was generated the same way under the same conditions. The primer for cDNA synthesis, flanked by a *Not*I site, was complementary to nt 18670 to 18698 of the vRNA; the second primer for PCR was homologous to the last 32 nt of the 5′ end and contained *Nco*I and *Eco*RI restriction sites. The leader was digested with *Sph*I and *Bam*HI and cloned into the vector pGEM3Zf(+). The trailer was digested with *Not*I and *Eco*RI and, afterward, was cloned into the pGEM-leader construct. Thus, the 3′ and 5′ ends (leader-trailer) were joined by a *Not*I site. The leader-trailer DNA was excised with *Nco*I, and the overlapping 5′ ends (5′-CATG) were partially filled with dCTP, using the Klenow fragment of DNA polymerase (Boehringer Mannheim; 20 min at 22°C). Thereafter, the remaining overlapping 5′ ends were removed by nuclease S1 (Boehringer Mannheim) treatment (final concentration, 66 mU/μl; 5 min at 37°C), and the fragment was inserted between the *Stu*I and *Sma*I sites of the transcription vector 2,0 (kindly provided by Andrew Ball, University of Alabama Medical School) (30). As a reporter gene, 668 nt of the CAT gene, flanked by *Not*I sites, was inserted between the leader and trailer sequences.

For generating negative-strand RNA, the 5′ end of the MBGV minigenome was positioned adjacent to the T7 RNA polymerase promoter and the 3′ end was followed by the hepatitis delta virus ribozyme sequence (Fig. 1A). To obtain MBGV-specific 3′ and 5′ ends, in vitro mutagenesis (Transformer site-directed mutagenesis kit; Clontech) or PCR mutagenesis followed by restriction fragment replacement was performed. In vitro transcription of the resulting DNA led to an RNA species with the following extreme genome ends: 3′-UC and GG-5′. The *Not*I site upstream of the CAT gene of the negative-strand construct was mutated to an *Nde*I site. Two vaccinia virus-specific transcription terminator regions (21) were inserted into the plasmid, the first (5′-T_5AT) between the MBGV leader and the start region of the CAT gene, using the *Nde*I restriction site, and the second (T_8) between the ribozyme sequence and the T7 RNA polymerase terminator. The resulting plasmid was designated 215 (Fig. 1A). Since the inserted vaccinia virus transcription terminator regions were found to be insufficient to prevent nonspecific CAT activity (see Results), double terminator motifs (5′-*Nde*I-T_5ATCGCAT$_5$CT-*Nde*I), generated with annealed primers, were inserted into the *Nde*I site. Due to there being a molar excess of the annealed primers during ligation, the terminator motifs were inserted four times in the plus-strand orientation and once in the minus-strand orientation; the resulting plasmid was designated 215_{term}. For production of positive-strand RNA, the fragments were cloned in the reverse direction (plasmid 2.1-CAT) (Fig. 1B). In vitro transcription resulted in a plus-strand RNA with the following genome ends: 3′-CC and GG-5′.

Plasmid 215 was used for construction of a copy-back minireplicon (Fig. 1C). Amplification of the last 105 nt of the 5′ end of MBGV was performed by RT-PCR with MBGV genomic RNA as a template, a forward primer complementary to nt 19004 to 19036 and containing an *Nde*I site, and a reverse primer homologous to nt 19087 to 19108 of the MBGV genome, which comprises the adjacent 38 nt of the ribozyme sequence, including an *Rsr*II restriction site. The 3′ leader was removed by digestion of plasmid 215 with *Nde*I and *Rsr*II and replaced by the PCR fragment. To provide the authentic NP-specific translational start region, the AUG start codon of NP, including 7 nt upstream and 5 nt downstream, was inserted in frame with the CAT gene. In vitro transcription of the constructed copy-back plasmid (cb-CAT [Fig. 1C]) resulted in a negative-stranded RNA whose first and last 105 nt were complementary.

In vitro transcription. Transcription of minigenome RNA was performed with an AmpliScribe T7 kit (Epicentre Technologies). One microgram of *Sal*I-digested purified minigenome DNA was in vitro transcribed in accordance with the supplier's protocol. After DNase I digestion (0.5 μg/μl), the RNA was purified by using an RNeasy kit (Qiagen) and eluted in 65 μl of H_2O containing 1 U of RNase inhibitor per μl. For RNA transfection (see below), usually 1 to 10 μl of purified RNA was used.

RNA transfection of MBGV-infected cells and passaging of CAT activity. Subconfluent E6 cells (approximately 10^6) were infected with MBGV strain Musoke at a multiplicity of infection (MOI) of 1 PFU per cell. At 1 h postinfection (p.i.), the cells were washed once with Dulbecco's modified Eagle medium (DMEM) and transfected with 5 to 10 μl of the recombinant RNA, using the transfection reagent DOTAP (Boehringer Mannheim), in a final volume of 2 ml of DMEM as described below. Twenty-four hours later, the medium was replaced by DMEM supplemented with 2% fetal calf serum, and 3 days later, the cells were assayed for CAT activity.

Supernatants of MBGV-infected and -transfected cells were clarified at 5 days p.i. and serially passaged to fresh E6 cells. The cells were harvested and processed for CAT assay and Northern blot analysis.

Combined DNA-RNA transfection of MVA-T7-infected cells. HeLa cells (10^6 per 7-cm^2 well) were infected with MVA-T7 at an MOI of 5 PFU per cell. At 1 h p.i., the cells were transfected with various amounts of plasmids encoding the nucleocapsid proteins of MBGV, as indicated in the text as well as in the figures, using the Lipofectin (Gibco BRL) transfection technique. At 3.5 h after transfection, the cells were washed three times with DMEM without fetal calf serum. After the last washing step, the cells were allowed to stand at 37°C for 15 min. During this time the RNA transfection reaction mixture was prepared by mixing 80 μl of 25 mM HEPES, pH 7.5, with 20 μl of DOTAP. In a separate tube, 1 to 10 μl of the in vitro-transcribed minigenome RNA was mixed with 25 mM HEPES, pH 7.5, to a final volume of 50 μl. The two solutions were combined, carefully mixed, and incubated for 10 min on ice. Two milliliters of DMEM was added, and the RNA transfection mixture was transferred to the DNA-transfected cells. The plates were further incubated for 48 to 72 h at 33°C.

CAT assay. CAT activity was determined by using 50 nCi of [^{14}C]chloramphenicol (Amersham Buchler)/sample in a standard assay (20). For CAT assays performed with MBGV-infected cells, lysates corresponding to 5×10^5 E6 cells were used. For CAT assays performed with vaccinia virus-infected cells, lysates corresponding to 10^5 HeLa cells (1/10 of a 7-cm^2 well) were used. Quantification of radioactivity was done with a Bio-Imaging Analyzer BAS-1000 (Fujifilm), using TINA software (Raytest).

RNA isolation and MCN treatment. (i) MBGV-infected cells. E6 cells (7.5×10^6) were infected with serially passaged MBGV containing artificial defective RNA particles at an MOI of 10^{-1} PFU per cell. Cells were lysed at 5 days p.i., and total cellular RNA was isolated by using an RNeasy kit (Qiagen) and subjected to Northern blot analysis.

(ii) Vaccinia virus system. HeLa cells grown in 7-cm^2 wells were infected with MVA-T7 and transfected with DNA as described above. At 2 days p.i., cells were washed two times with phosphate-buffered saline, scraped into the washing buffer, and pooled (three wells). After centrifugation for 10 min at 3,000 rpm in a Heraeus Megafuge 1.0 centrifuge at 4°C, the pellets were resuspended in 200 μl of micrococcal nuclease (MCN) buffer (10 mM NaCl, 10 mM Tris [pH 7.5], 1.5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride) (16), sheared, and sonicated for 1 min. A 50-μl aliquot of each sample was processed directly for RNA isolation, using an RNeasy kit (Qiagen). In accordance with the protocol of Fearns et al. (16), the remaining 150 μl of each lysate was treated with 3 μl of MCN (Boehringer Mannheim; 15 U/μl) for 75 min at 30°C. Subsequently, RNA was isolated by using an RNeasy kit.

(iii) Oligo(dT) purification. Total cellular RNA corresponding to three 7-cm^2 wells of MVA-T7-infected and transfected HeLa cells was isolated at 2 days p.i. as described above. Polyadenylated RNA was purified from total RNA by using oligo(dT) cellulose (microcrystalline; New England Biolabs). Unbound and

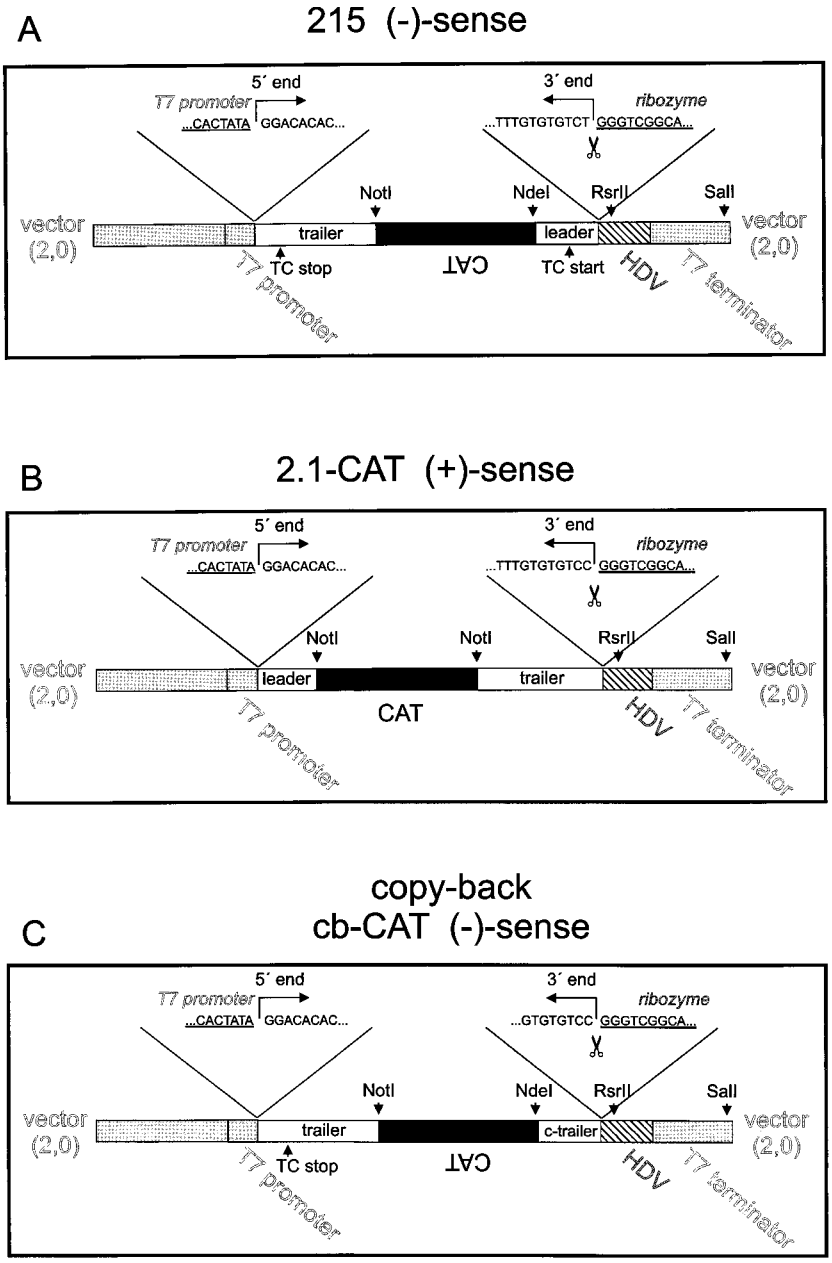


FIG. 1. Construction of artificial defective RNAs of MBGV. The minigenomes were inserted in transcription vector 2,0 (gray) between the T7 RNA polymerase promoter and the hepatitis delta virus ribozyme (hatched). For in vitro transcription, plasmids were linearized by using the *Sal*I restriction site (right side). (A) Diagram of negative-sense minigenomic cDNA 215, consisting of 439 nt of the 5′ trailer (white) adjacent to the T7 RNA polymerase promoter, 668 nt of the CAT gene in a negative-sense orientation (black), and 106 nt of the 3′ leader (white) adjacent to the ribozyme. Above the scheme are indicated the boundary between the T7 RNA polymerase promoter sequence (underlined) and the 5′ end of the minigenome (negative-sense orientation) (left side) and the boundary between the ribozyme sequence (underlined) and the 3′ end of the minigenome (right side). The CAT gene is flanked by *Not*I and *Nde*I restriction sites. (B) Diagram of positive-sense minigenomic cDNA 2.1-CAT, consisting of 106 nt of the 3′ leader (white) adjacent to the T7 RNA polymerase promoter, the CAT gene in a positive-sense orientation (black), and 439 nt of the 5′ trailer adjacent to the ribozyme sequence (white). The boundaries between the T7 RNA polymerase promoter and the ribozyme sequence (underlined), respectively, and the MBGV-specific sequences are indicated. MBGV-specific sequences are shown in the plus-strand orientation. The CAT gene is flanked by *Not*I restriction sites. (C) Diagram of the cDNA coding for the copy-back-type negative-stranded minigenome cb-CAT. The minigenome consists of 439 nt of the 5′ trailer (white) adjacent to the T7 RNA polymerase promoter, the CAT gene in a negative-sense orientation (black), and, adjacent to the ribozyme sequence, 105 nt complementary to the last 105 nt of the trailer (designated as c-trailer; white), which serves as the leader region. The boundaries between the T7 RNA polymerase promoter and the ribozyme sequence (underlined), respectively, and the MBGV-specific sequences are indicated. MBGV-specific sequences are shown in the negative-sense orientation. The CAT gene is flanked by *Not*I and *Nde*I restriction sites. TC start, transcription start site of the NP gene, spanning nt 49 to 60 of the leader region; TC stop, transcription stop site of the L gene, spanning nt 353 to 363 of the trailer region (27). Transcription start and stop sites are indicated only for negative-stranded minigenomes. The cleavage site of the ribozyme is symbolized by a pair of scissors.

eluted bound RNA were ethanol precipitated and subjected to Northern blot analysis.

Northern blot analysis. RNA samples were separated on 1.5% agarose gels containing 0.44 M formaldehyde and blotted onto positively charged nylon membranes (Boehringer Mannheim) for 90 min with a vacuum blotter (Appligene), using 3 M NaCl–8 mM NaOH as the transfer buffer (7). Then, RNA was fixed for 3 min by UV cross-linking. Hybridization was performed as described by Grosfeld et al. (21). Briefly, membranes were prehybridized for 6 h at 65°C in a

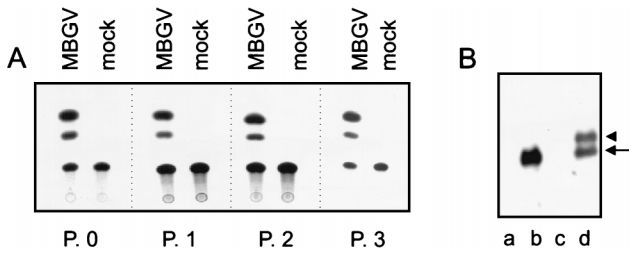


FIG. 2. Rescue of minigenomic RNA in MBGV-infected cells. (A) Supernatants of MBGV- or mock-infected E6 cells (mock) that were transfected with the negative-sense RNA 215 were serially passaged to fresh E6 cells. At 5 days p.i., the cells were harvested and assayed for CAT activity. The number of passages is designated as P.0 (transfection) to P.3 (third passage). (B) E6 cells were infected with MBGV (lane a) or with serially passaged MBGV (P.3) containing artificial defective RNA particles 215 (lane b) or were not infected (lane c). At 5 days p.i., total cellular RNA was isolated and subjected to Northern blot analysis. As the probe, a digoxigenin-labeled positive-sense riboprobe DIG-2.1-CAT was used. As a control, in vitro-transcribed RNA 215 was separated on the gel (lane d). The arrowhead marks the position of uncleaved RNA 215 containing the ribozyme; the arrow marks the position of cleaved RNA 215 after removal of the ribozyme.

solution consisting of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 0.5 mg of denatured, fragmented salmon sperm DNA per ml. After addition of 3 μl of digoxigenin-labeled riboprobe (see below), hybridization was done overnight at 65°C. Filters were washed for 5 min at room temperature in 0.1× SSC–0.1% SDS and then for 2 h at 65°C in the same washing solution. Chemiluminescent detection of the hybridized probe was performed with CDP-Star in accordance with the supplier's manual (Boehringer Mannheim). For preparation of positive-stranded riboprobes, 1 μg of the positive-sense minigenomic DNA 2.1-CAT was digested with *Hin*dIII and transcribed in vitro, using T7 RNA polymerase and the Dig RNA Labeling Kit (Boehringer Mannheim). For preparation of negative-sense riboprobes, 1 μg of pBluescript II KS vector containing the CAT gene (BS/CAT) was cut with *Sac*I and in vitro transcribed as described above. After purification, RNA probes were eluted in 100 μl of H_2O.

Western blot analysis. HeLa cells (1.4×10^6) were infected with MVA-T7 and transfected with plasmids encoding the nucleocapsid protein genes of MBGV as described above. After incubation at 37°C overnight, the cells were washed twice with phosphate-buffered saline and then scraped into 500 μl of Triton lysis buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 55 mM Tris-HCl, (pH 7.8), 200 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 5% (vol/vol) Trasylol, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (10% gel; 10 μl per lane). Western blot analysis was performed as described elsewhere (2). For detection of NP, a monoclonal antibody raised against NP and diluted 1:10,000 was used. For detection of VP35, a monoclonal antibody raised against VP35 (a gift of Anthony Sanchez, Centers for Disease Control and Prevention, Atlanta, Ga.) and diluted 1:40,000 was used. For detection of VP30, a guinea pig anti-VP30 serum diluted 1:15,000 was used (gift of Heinz Feldmann, Institute of Virology, Marburg, Germany). Secondary antibodies coupled with peroxidase were diluted 1:100,000 and were detected with Super Signal Ultra reagent (Pierce). Quantitation of chemiluminescence signals was done with a Bio-Imaging Analyzer BAS-1000 (Fujifilm), using TINA software (Raytest).

RESULTS

Rescue of an artificial minigenome in MBGV-infected cells. A synthetic defective MBGV-specific cDNA containing the 3′ and 5′ ends of MBGV RNA and lacking all viral genes, which were replaced by the CAT gene, was constructed (Fig. 1A). By in vitro transcription of this construct, an artificial minigenome with negative polarity (designated as 215) was synthesized. Correct 3′ ends were generated by autolytic cleavage of the hepatitis delta virus ribozyme (30). To check whether this RNA construct could serve as a template for the MBGV replication complex, MBGV-infected E6 cells were transfected with RNA 215. At 3 to 4 days p.i., the cells were lysed and CAT activity was determined (Fig. 2A). Expression of the CAT gene was completely dependent on MBGV infection; uninfected cells transfected with the minigenome (Fig. 2A, mock) were negative for CAT activity. To prove that the artificial minigenome was replicated and could be passaged, clarified supernatants of MBGV-infected cells transfected with the artificial minigenome were used to infect fresh E6 cells. At 5 days p.i., cells were lysed to measure CAT activity and supernatants were passaged again to fresh cells. After three passages, CAT activity was still detected, without significant changes in signal strength, indicating that in fact the artificial MBGV RNA could serve as a template for replication, transcription, and encapsidation (Fig. 2A). These results were confirmed by Northern blot analysis. RNA isolated from cells of the third passage was hybridized with a positive-sense riboprobe. As shown in Fig. 2B, a specific RNA band (lane b) which was similar in size to the cleaved input RNA (lane d, arrow) was detected. Unexpectedly, an increase in CAT activity as a consequence of a preferential replication of the small minigenome was not detected.

Expression of MBGV nucleocapsid proteins in MVA-T7-infected HeLa cells. A prerequisite for developing an artificial replication and transcription system for MBGV was the expression of recombinant nucleocapsid proteins. Plasmids encoding NP, VP35, and VP30 under the control of the T7 RNA polymerase promoter were used to transfect MVA-T7-infected HeLa cells, and protein expression was examined by Western blot analysis. NP, VP35, and VP30 were transiently expressed and migrated on SDS-polyacrylamide gels with the same mobility as the authentic MBGV proteins (data not shown). Since antibodies recognizing L were not suitable to detect weakly expressed L, its expression was verified only by its function (see below).

CAT gene expression mediated by MBGV proteins. Next, a study designed to determine which of the four nucleocapsid proteins were essential for MBGV transcription and replication was done. HeLa cells were infected with MVA-T7 and transfected with plasmid-encoded nucleocapsid genes and the plasmid coding for the minigenomic RNA 215. Unfortunately, DNA transfection of plasmids coding for MBGV-specific negative-sense minigenomes containing the CAT gene led to vaccinia virus-driven CAT gene expression. Grosfeld et al. (21), working with RSV-specific minigenomes, determined that insertion of several vaccina virus-specific transcription termination motifs at different positions in the plasmid eliminated background CAT activity. The MBGV-specific plasmid 215 also contains two vaccinia virus-specific transcription terminators, but these did not prevent nonspecific CAT activity (data not shown).

To abolish nonspecific CAT activity, MVA-T7-infected cells were transfected first with plasmid-encoded nucleocapsid genes and then, 3.5 h later, with in vitro-transcribed negative-stranded RNA 215. At 3 days p.i., the cells were harvested and CAT activity was determined. Since NP is the most abundant protein of the nucleocapsid complex (3), the mixture of plasmids first employed for these experiments contained 2 μg of pT/NP, 0.5 μg of pT/VP35, 0.5 μg of pT/VP30, and 1 μg of pT/L. Under these conditions, no reporter gene expression could be detected (Fig. 3A, lane 6). However, when the amount of pT/NP was decreased to 100 ng, CAT gene expression was observed. Omission of NP, VP35, or L totally abrogated CAT activity, whereas omission of VP30 had no influence on CAT gene expression (Fig. 3A). These data indicate that NP, VP35, and L are the key components of MBGV-specific reporter gene expression.

Titration experiments performed with a fixed amount of pT/NP (100 ng) and various concentrations of pT/VP35, pT/VP30, and pT/L revealed that the amounts of VP35 and L input DNA were not very critical (Fig. 3B, VP35 and L). Optimal results were obtained by using 0.5 to 1 μg of pT/VP35 and 1 μg of pT/

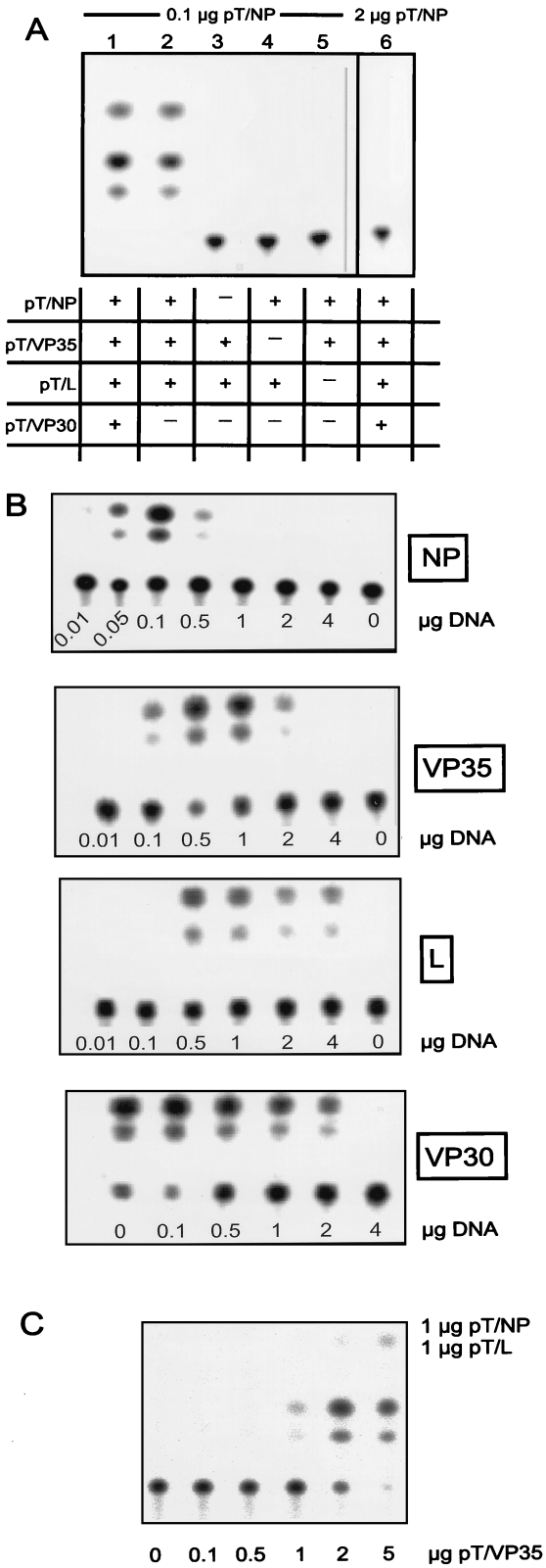


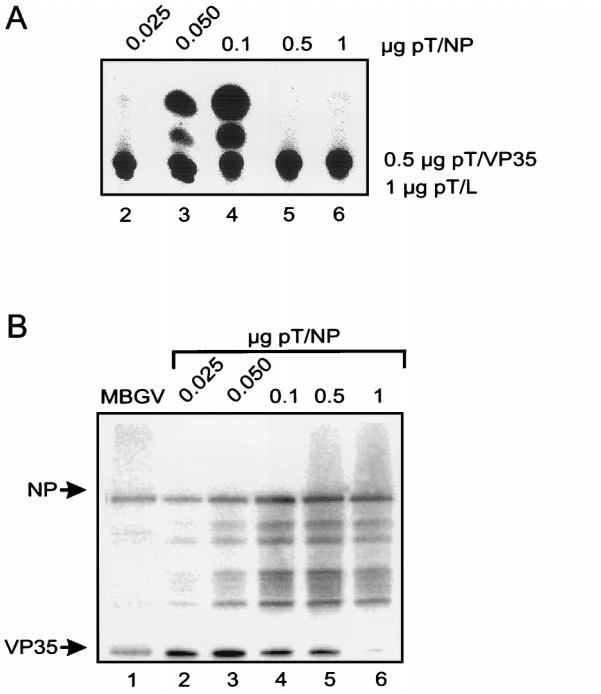
FIG. 3. Reporter gene expression in cells transfected with plasmids encoding MBGV nucleocapsid proteins and transfected with negative-sense MBGV-specific minigenome 215. HeLa cells were infected with MVA-T7, transfected with pT/NP, pT/VP35, pT/L, and/or pT/VP30, and subsequently transfected with RNA 215. At 3 days p.i., cells were lysed, CAT activity was determined and acetylated products were separated by thin-layer chromatography. (A) DNA transfection was performed with 0.1 (lanes 1 to 5) or 2 (lane 6) μg of pT/NP, 0.5 μg of pT/VP35, 1 μg of pT/L, and/or 0.5 μg of pT/VP30 as indicated. (B) Titration of plasmids encoding the different nucleocapsid proteins. Titration experiments were performed with fixed amounts of three of the plasmids encoding nucleocapsid proteins (pT/NP, 0.1 μg; pT/VP35, 0.5 μg; pT/L, 1 μg; pT/VP30, 0.5 μg) and various amounts of one of the plasmids as indicated at the right side of each panel. (C) Titration of pT/VP35 at large amounts of NP input DNA. DNA transfection was performed with 1 μg of pT/NP, 1 μg of pT/L, and various amounts of pT/VP35 as indicated.

L. Addition of pT/VP30 up to 2 μg of input DNA did not lead to suppression of CAT activity (Fig. 3B, VP30). The amount of NP input DNA profoundly affected CAT gene expression (Fig. 3B, NP). CAT activity was observed only with small amounts of pT/NP. Above 0.5 μg of NP input DNA, CAT activity was completely abrogated.

The question of whether it was the absolute amount of NP that was critical for the system or rather the ratio between NP and another nucleocapsid protein arose. To check whether the NP/VP35 ratio influences CAT gene expression, the amounts of both input DNAs were increased in parallel. As shown in Fig. 3C, reporter gene expression was observed even at 1 μg of pT/NP if the amount of pT/VP35 was increased to 5 μg, indicating that it is not the absolute amount of NP that is critical but rather the ratio of NP to VP35.

The NP/VP35 ratio in the functional artificial replication system reflects the situation in MBGV-infected cells. Since it was shown that the NP/VP35 input DNA ratio had to be 1:5 to mediate MBGV-dependent reporter gene expression, it was of interest to determine if this ratio led to NP and VP35 protein concentrations which reflected the situation in MBGV-infected cells. MVA-T7-infected HeLa cells were transfected with fixed amounts of pT/VP35 (0.5 μg) and pT/L (1 μg) and various concentrations of pT/NP (25 ng to 1 μg). Then, DNA-transfected cells were transfected with RNA 215. Cells were harvested, and the cell lysates were split and subjected to CAT assay and Western blot analysis (Fig. 4). With regard to CAT gene expression, the strongest signal was obtained at a pT/NP-to-pT/VP35 ratio of 1:5 (Fig. 4A, lane 4). When the ratio was shifted to 1:20 or lower (lane 2) or 1:1 or higher (lanes 5 and 6), reporter gene expression was totally abrogated. To determine the NP/VP35 ratio at the protein level, Western blot analyses were carried out with the same lysates and in parallel with lysates of MBGV-infected cells, using NP- and VP35-specific monoclonal antibodies. As shown in Fig. 4B, NP expression increased in response to increasing amounts of transfected pT/NP. The increase in signal strength was linear in the range of 25 to 100 ng of pT/NP and reached a plateau above 100 ng. The ratio of the chemiluminescence signals of NP and VP35 in MBGV-infected cells was 6:1 (Fig. 4B, lane 1). A similar value (8.5:1) was detected when the ratio of the plasmids encoding NP and VP35 was 1:5 (lane 4). Under these conditions, the replication system was functional. When the ratio of pT/NP to pT/VP35 was raised to 2:1 (lane 6), expression of VP35 was strongly suppressed, leading to an NP/VP35 protein ratio of 132:1. From these data taken together, it is evident that the NP/VP35 ratio in MBGV-infected cells is in the range of that observed for the functional artificial replication system.

Replication and encapsidation of MBGV minireplicons. To determine whether the minireplicons were transcribed and/or replicated by recombinant expressed proteins, RNA detection assays were performed. Using the combined DNA-RNA transfection protocol, no MBGV-specific RNA was detected (data not shown). Therefore, the transfection protocol was changed. MVA-T7-infected cells were transfected with plasmids coding



sample	1	2	3	4	5	6
NP	28,296	17,002	35,573	68,661	96,450	83,657
VP35	4,592	9,708	13,874	8,090	6,791	631
ratio NP:VP35	6	1.7	2.6	8.5	14	132

FIG. 4. Determination of the NP/VP35 ratio. HeLa cells seated in 7-cm^2 wells were infected with MVA-T7 and transfected with 0.5 μg of pT/VP35, 1 μg of pT/L, and various amounts of pT/NP as indicated. At 3.5 h after DNA transfection, cells were transfected with RNA 215. After incubation at 37°C overnight, the cells were harvested. Cell lysates were split and subjected to CAT assay and Western blot analysis. (A) CAT assay performed with 50% of each lysate. (B) Western blot analysis. Lysates corresponding to 3×10^4 cells (lanes 2 to 6) and a lysate of MBGV-infected E6 cells (lane 1) were analyzed by Western blotting with monoclonal antibodies directed against NP (dilution, 1:10,000) and VP35 (dilution, 1:40,000). Chemiluminescence signals specific for NP and VP35 expression were quantified (given in relative densitometer units), and the NP/VP35 ratio was determined (table at bottom). For quantification of NP, degradation products were included. The antibody concentration was adjusted so that the chemiluminescence signals received could be quantitatively evaluated. The signal strength ratios therefore do not necessarily reflect the actual protein ratios.

for the supporting proteins and the plasmid encoding the positive-sense minireplicon 2.1-CAT. At 2 days p.i., the cells were lysed and total cellular RNA was isolated, treated with MCN, and analyzed by Northern hybridization with a positive-sense riboprobe. Minigenomic negative-sense RNA which was resistant to MCN treatment was detected, indicating that the RNA was replicated and encapsidated (Fig. 5A, lane 3). The protected RNA species comigrated with in vitro-transcribed cleaved RNA 215, which was used as marker (lane 1, arrow). As has been shown for reporter gene expression, NP, VP35, and L were sufficient for replication. When the amount of NP input DNA was increased, replication was totally inhibited (Fig. 5B). Addition of pT/VP30 was not necessary to support replication, thus confirming the results of the CAT assays.

Transcription of MBGV minigenomes. As mentioned above, DNA transfection with plasmids encoding negative-stranded MBGV minigenomes led to vaccinia virus-driven synthesis of CAT mRNA because the CAT gene was transcribed by vaccinia virus DNA-dependent RNA polymerases. Like MBGV, vaccinia virus also has the capacity for polyadenylation (19), making it impossible to distinguish mRNA transcribed by vaccinia virus from mRNA transcribed by MBGV proteins. For detection of MBGV-specific transcription, it was necessary to use a minigenomic DNA which was not transcribed by vaccinia virus RNA polymerases. Since transfection with plasmid 215 (Fig. 1A), containing two vaccinia virus-specific transcription terminators at different sites, still led to nonspecific CAT activity (see above), additional vaccina virus transcription terminator regions were inserted between the MBGV leader sequence and the CAT gene. After insertion of an array of eight terminators in the plus-strand orientation, background CAT gene expression was strongly reduced but not totally abolished (data not shown). However, the resulting plasmid (215_{term}) was used for cotransfection of MVA-T7-infected HeLa cells with pT/NP, pT/VP35, pT/L, and pT/VP30. At 2 days p.i., cells were harvested and the lysates were split and either treated with MCN or not treated. After oligo(dT) purification, the RNA was subjected to Northern blot analysis with a negative-sense digoxigenin-labeled riboprobe. As shown in Fig. 6A, positive-sense RNA of the predicted size was detected in the unbound and undigested RNA fraction. The appearance of the specific RNA band was exclusively dependent on the presence of NP, VP35, and L (Fig. 6A, lane 3), whereas background signals were probably due to vaccinia virus-specific RNA synthesis with plasmid 215_{term} as the template (Fig. 6A, lane 4). A small part of the unbound RNA fraction was shown to be nuclease resistant, as had been implied for replicated and encapsidated plus-strand RNA (Fig. 6B, lanes 1 to 3). In the presence of larger amounts of VP30, replication of the minigenome seemed to be inhibited (Fig. 6, lanes 1). However, this effect might be due to an overexpression of VP30 at 500 ng of VP30 input DNA, since smaller amounts of VP30 (25 ng of input DNA) did not influence the efficiency of replication (Fig. 6B, lane 2). Polyadenylated MBGV-specific RNA was detected only when the samples were not nuclease treated (Fig. 6, lanes 5 to 8), indicating that this RNA species was not encapsidated, as had been predicted for mRNA. The two RNA species, replicated plus-strand RNA and mRNA, were similar in size. Like replication, transcription of the used monocistronic minigenome was dependent on the presence of NP, VP35, and L as supporting proteins (Fig. 6A, lane 7). When L was omitted, specific signals could not be detected (Fig. 6, lanes 4 and 8). Interestingly, VP30 did not influence the synthesis of polyadenylated RNA (Fig. 6A, lanes 5 and 6).

CAT activity reflects transcription. As mentioned above, NP, VP35, and L were sufficient to support MBGV-specific reporter gene expression. When MVA-T7-infected cells, which did not express MBGV proteins, were transfected with positive-sense minigenome RNA, CAT activity was detected, indicating that this RNA could serve as a messenger (Fig. 7A, right panel). Since MBGV-specific replication involves plus-strand RNA intermediates, the question of whether the detected CAT activity generated by MBGV proteins was due entirely to mRNA synthesis—i.e., transcription—or partly to synthesis of plus-strand minigenomes—i.e., replication—arose. To address this question, an artificial copy-back minireplicon in which the leader region of minigenome 215 was replaced by 105 nt complementary to the 5′ end of MBGV genome was constructed (cb-CAT [Fig. 1C]). In vitro transcription of cb-CAT resulted in a negative-sense RNA lacking MBGV-specific transcrip-

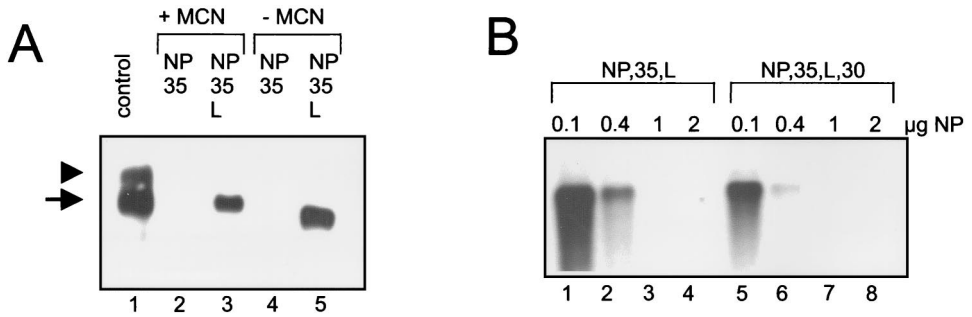


FIG. 5. Replication of MBGV-specific positive-sense minigenome 2.1-CAT. (A) HeLa cells were infected with MVA-T7 and transfected with 0.1 μg of pT/NP, 0.5 μg of pT/VP35, 1 μg of pT/L, and 0.5 μg of plasmid DNA 2.1-CAT (lanes 3 and 5). As a negative control, pT/L was omitted (lanes 2 and 4). At 2 days p.i., the cells were lysed and the lysates were either treated with MCN (+) (lanes 2 and 3) or were not treated (−) (lanes 4 and 5). Then, total RNA was isolated and subjected to Northern hybridization, using the positive-sense digoxigenin-labeled riboprobe DIG-2.1-CAT. As a control, in vitro-transcribed negative-sense RNA 215 was used. The arrowhead indicates uncleaved RNA 215 containing ribozyme; the arrow indicates cleaved RNA 215. (B) HeLa cells were infected with MVA-T7 and transfected with various amounts of pT/NP as indicated, along with 0.5 μg of pT/VP35, 1 μg of pT/L, and 0.5 μg of plasmid DNA 2.1-CAT, without (lanes 1 to 4) or with (lanes 5 to 8) 0.5 μg of pT/VP30. At 2 days p.i., cells were lysed and treated with MCN. Total cellular RNA was isolated and subjected to Northern hybridization with the positive-sense digoxigenin-labeled riboprobe DIG-2.1-CAT.

tional start signals upstream of the CAT gene. When MBGV-infected cells were transfected with cb-CAT RNA, the CAT gene was not expressed (Fig. 7A, left panel). Transfection of control RNA 215 led to CAT gene expression. The same result was obtained when MVA-T7-infected cells expressing MBGV nucleocapsid proteins were transfected with cb-CAT RNA; i.e., CAT gene expression was not observed (data not shown). However, Northern hybridization carried out with RNA isolated from MVA-T7-infected and pT/NP, pT/VP35, pT/L, and cb-CAT DNA-transfected HeLa cells demonstrated that cb-CAT RNA was replicated (Fig. 7B). Negative-strand as well as positive-strand RNA was detected. Both RNA species were resistant to MCN treatment. Although plus-strand RNA had been synthesized, no CAT activity was observed. This result suggested that CAT activity generated by MBGV proteins was due to transcription and not to replication. To prove that the CAT gene of the cb-CAT plasmid could serve as template for transcription and translation, a cassette containing the sequences between the ribozyme and the T7 RNA polymerase promoter was cloned into the vector pBluescript II KS under the control of the T3 RNA polymerase promoter. In vitro transcription by T3 RNA polymerase resulted in a positive-sense RNA (BS/cb-CAT) which was used for transfection of MVA-T7-infected cells (Fig. 7A, right panel). As a control, plus-stranded 2.1-CAT RNA and negative-stranded RNA cb-CAT were employed. Since BS/cb-CAT transfection led to CAT gene expression, it was evident that cb-CAT contained a functional CAT gene, thus supporting the hypothesis that reporter gene expression indeed reflects MBGV-specific transcription.

DISCUSSION

In this article, a reverse genetic system for MBGV is described. It was shown that three of the four MBGV nucleocapsid proteins, NP, VP35, and L, are sufficient to support replication and transcription of monocistronic minireplicons. For various rhabdo- and paramyxoviruses, it is also known that N (NP), P, and L are the minimum protein requirements for replication (10, 12, 14, 21, 29, 30, 32, 38). For most rhabdo- and paramyxoviruses, the same three proteins are sufficient to support transcription. Pneumoviruses, however, need an additional transcription factor (8). Comparison of the primary sequences of MBGV proteins to those of other NNS RNA viruses clearly revealed that MBGV NP and L are homologous to the N (NP) and L proteins of paramyxo- and rhabdoviruses (28, 33). Since P proteins are not well conserved among NNS RNA viruses, it is not surprising that the amino acid sequence of VP35 was not found to be homologous to any P protein sequence. However, VP35 exhibits some typical features of P proteins: it is encoded by the second gene of the viral ge-

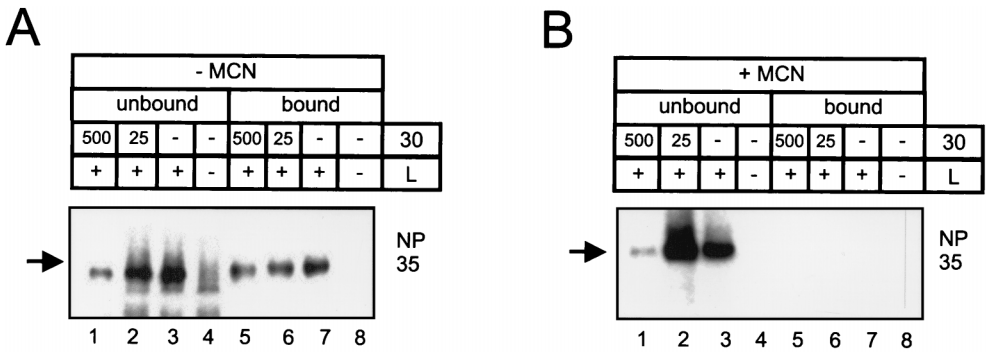


FIG. 6. Transcription of MBGV-specific negative-sense minigenome 215_{term}. HeLa cells were infected with MVA-T7 and transfected with 0.1 μg of pT/NP, 0.5 μg of pT/VP35, either 25 ng of pT/VP30 (lanes 2 and 6) or 500 ng of pT/VP30 (lanes 1 and 5), 2 μg of plasmid DNA 215_{term}, and 1 μg of pT/L. As a negative control, pT/L was omitted (lanes 4 and 8). At 2 days p.i., the cells were lysed and either treated with MCN (+) or not treated (−). Total cellular RNA was isolated and subjected to oligo(dT) purification. Northern blot analysis was performed with the negative-sense digoxigenin-labeled riboprobe DIG-BS/CAT. The blot exposure time was 30 s (A) or 30 min (B). unbound, RNA fraction which did not bind to oligo(dT) cellulose; bound, RNA fraction that bound to oligo(dT) cellulose; 25, 25 ng of pT/VP30; 500, 500 ng of pT/VP30. The arrows indicate the position of the replicated and transcribed RNA.

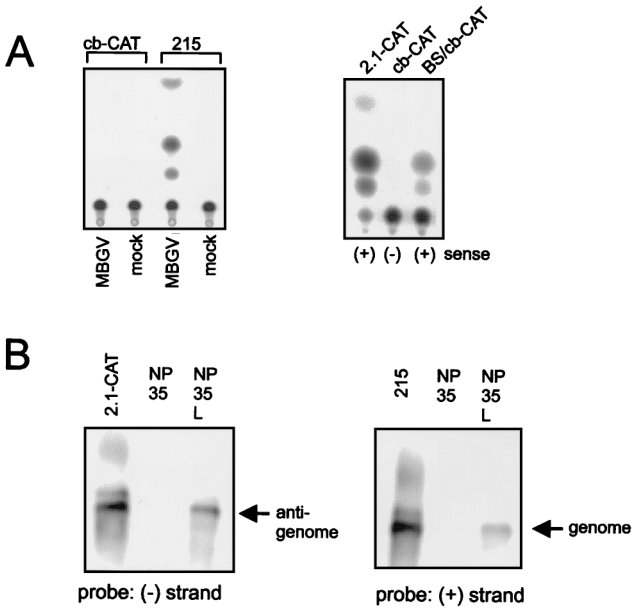


FIG. 7. CAT gene expression reflects transcription. (A) Determination of CAT gene expression of cells transfected with copy-back RNA cb-CAT. Left panel: uninfected (mock) or MBGV-infected E6 cells were transfected with RNA cb-CAT or RNA 215. At 3 days p.i., cells were lysed and CAT activity was determined. Right panel: HeLa cells were infected with MVA-T7 and transfected with in vitro-transcribed positive-sense RNA 2.1-CAT, negative-sense RNA cb-CAT, or positive-sense RNA BS/cb-CAT. At 24 h p.i., the cells were lysed and assayed for CAT activity. (B) Replication of copy-back minigenome cb-CAT. HeLa cells were infected with MVA-T7 and transfected with 0.1 μg of pT/NP, 0.5 μg of pT/35, 1 μg of pT/L, and 0.5 μg of plasmid DNA cb-CAT (lanes NP/35/L). As a negative control, pT/L was omitted (lanes NP/35). At 2 days p.i., the cells were lysed and treated with MCN. Duplicate Northern blots were prepared from total cellular RNA, with either in vitro-transcribed positive-sense RNA 2.1-CAT (left panel) or in vitro-transcribed negative-sense RNA 215 (right panel) as a control. Both blots were subjected to Northern hybridization, using either the negative-sense digoxigenin-labeled riboprobe DIG-BS/CAT (left panel) or the positive-sense digoxigenin-labeled riboprobe DIG-2.1-CAT (right panel). The positive-stranded antigenome and the negative-stranded genome are marked by arrows.

nome, and it has been shown to be a nucleocapsid protein that interacts with NP and L (1). On the other hand, there is one striking difference between the rhabdo- and paramyxovirus P proteins: MBGV VP35 is only very weakly phosphorylated. Moreover, for Ebola virus, it has been reported that only NP and VP30 are phosphorylated (15). Our observation that VP35 is essential for MBGV replication clearly indicates that this protein is indeed the filovirus-specific P analogue. Therefore, it is proposed that VP35 be renamed P.

Titration experiments performed with the supporting plasmids demonstrated that the amount of NP input DNA was very critical for the system. When large amounts of NP DNA were used, CAT gene expression was completely suppressed. Since reporter gene expression probably reflected transcription (see below), one could argue that small amounts of NP might be sufficient for transcriptional activity but not for replication. However, the same effect was found for MBGV-specific replication as could be shown by Northern blot analysis. This was surprising because, first, NP is the most abundant nucleocapsid protein and, second, it was shown for other NNS RNA viruses that large amounts of NP DNA did not abrogate replication and/or transcription. For vesicular stomatitis virus, it was demonstrated that increasing amounts of N DNA in the range of 15 to 25 μg at fixed amounts of NS (10 μg) and L (5 μg) plasmid DNA did not affect replication of defective interfering particle RNA (31). At larger amounts of N DNA (up to 50 μg), replication was decreased but not abrogated. Also, titration of the levels of N, P, and L plasmids of RSV showed that reporter gene expression was maximal when the N and P plasmids were present in equal amounts, but increasing amounts of N input DNA did not reduce CAT gene expression and replication of minigenomes substantially (21). Comparable results have been obtained for Sendai virus (24). Titration of NP DNA was performed in the range of 0.25 to 2 μg (1 μg of L DNA, 0.5 μg of P DNA) without the loss of reporter gene expression. Interestingly, as found with MBGV, reporter gene expression was affected by changing the relative amounts of the supporting plasmids. In contrast to MBGV, the amount of P DNA was particularly critical (24). Additional titration experiments performed with MBGV NP and VP35 clearly indicated that it was not the absolute amount of NP that was critical for the system but rather the NP/VP35 ratio. The best results were obtained when the NP/VP35 plasmid ratio was 1:5. This ratio was observed to be optimal for transcription as well as replication. Western blot analysis of cell lysates obtained from MBGV-infected cells revealed an NP/VP35 ratio of 6:1. This value does not reflect absolute protein amounts and is due to the chosen antibody dilutions. Simultaneous Western blot analyses carried out with MVA-T7-infected and NP, VP35, and L plasmid-transfected cells resulted in a similar NP/VP35 ratio when the plasmid ratio was 1:5.

For various NNS RNA viruses it has been said that N (NP) tends to self-assemble. In this form the protein is inactive. Interaction with P stabilizes the soluble form of N (NP), thus maintaining the replication complex in an active state (4, 11, 23, 26). MBGV NP also has a strong tendency to self-aggregate which is reflected in the formation of large inclusion bodies intracellularly by recombinant NP (1). It is hypothesized that the interaction between NP and VP35 is critical for keeping NP functional. A defined NP/VP35 input DNA ratio might reflect the stoichiometry of the NP-VP35 complex.

An artificial copy-back minireplicon lacking MBGV-specific transcriptional start sites was found to be a useful tool to differentiate between replication and transcription. This copy-back minigenome was shown to be replicated by MBGV proteins but did not lead to CAT gene expression. When defective minireplicons with authentic 3′ and 5′ ends—i.e., with an active transcriptional start site—were used for the same experiments, replicated RNA and, in parallel, CAT activity were detected. These data clearly indicated that CAT gene expression was induced by MBGV-specific mRNA synthesis and not by positive-sense minigenomes. Thus, CAT activity reflected transcription and not replication. The fact that reporter gene expression is linked to viral transcription has also been noted by Kuo et al. (25). RSV minigenomes lacking transcriptional start or stop signals induced only minimal amounts of CAT activity compared to that induced by the nonmutated minireplicon. The two mutants, however, were replicated with the same efficiency as the nonmutated minigenome. For the bunyavirus system it has also been postulated that CAT activity is due to transcription (13). Since MBGV positive-sense input RNA was accepted as a messenger and hence was translated, the question of why replicated plus-stranded RNA was not translated arose. One possible explanation is that encapsidation of replicated RNA interfered with translation.

For RSV it has been reported that the fourth nucleocapsid protein, M2, is an essential cofactor for viral transcription, acting as an elongation factor (8) and as an antiterminator (22) during viral transcription. While M2 influences the synthesis of mRNA, reporter gene expression is not dependent on the presence of M2 (21), i.e., on the proper elongation of the mRNA. Filoviruses also possess an additional nucleocapsid

protein (VP30) which is tightly linked to the ribonucleoprotein core (1, 15) and which is highly phosphorylated. In this paper, we present evidence that VP30 is not necessary for replication of monocistronic MBGV-specific minigenomes and, in contrast to M2, is not essential for synthesis of full-length transcripts. Since our experiments were performed with monocistronic minigenomes, it must be elucidated whether VP30 plays a role during replication and transcription of polycistronic RNA.

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